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**Latent-transforming growth factor  $\beta$ -binding protein 1/Transforming growth factor  $\beta$ 1 complex drives antitumoral effects upon ERK5**

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Latent-transforming growth factor  $\beta$ -binding protein 1/Transforming growth factor  $\beta$ 1 complex drives antitumoral effects upon ERK5 targeting in melanoma

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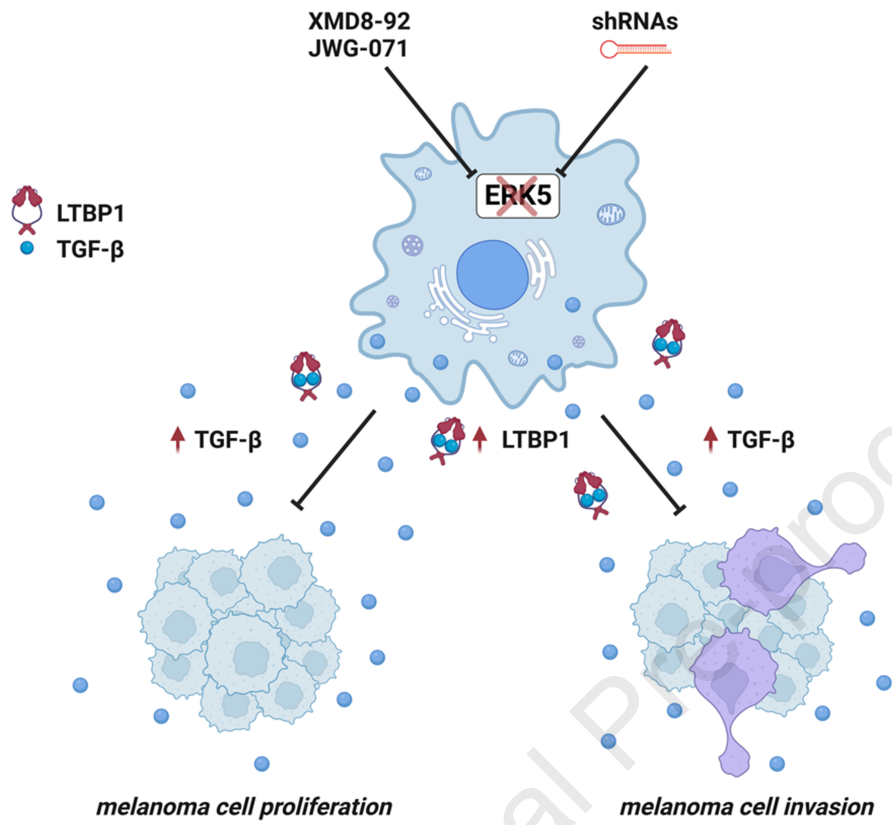
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## ERK5 targeting in melanoma



1           **Latent-transforming growth factor  $\beta$ -binding protein 1/Transforming growth factor**  
2            **$\beta$ 1 complex drives antitumoral effects upon ERK5 targeting in melanoma**

3  
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**42 Abstract**

43 Melanoma is the deadliest skin cancer, with a poor prognosis in advanced stages. Available  
44 treatments have improved the survival, although long-term benefits are still unsatisfactory. The  
45 mitogen-activated protein kinase ERK5 promotes melanoma growth, and ERK5 inhibition  
46 determines cellular senescence and the senescence-associated secretory phenotype. Here, latent-  
47 transforming growth factor  $\beta$ -binding protein 1 (LTBP1) mRNA was found to be upregulated in A375  
48 and SK-Mel-5 BRAFV600E melanoma cells after ERK5 inhibition. In keeping with a key role of  
49 LTBP1 in regulating transforming growth factor  $\beta$  (TGF- $\beta$ ), TGF- $\beta$ 1 protein levels were increased  
50 in lysates and conditioned media of ERK5-knock down (KD) cells, and were reduced upon LTBP1  
51 KD. Both LTBP1 and TGF- $\beta$ 1 proteins were increased in melanoma xenografts in mice treated with  
52 the ERK5 inhibitor XMD8-92. Moreover, treatment with conditioned media from ERK5-KD  
53 melanoma cells reduced cell proliferation and invasiveness, and TGF- $\beta$ 1-neutralizing antibodies  
54 impaired these effects. In silico datasets revealed that higher expression levels of both LTBP1 and  
55 TGFB1 mRNA are associated with better overall survival of melanoma patients, and that increased  
56 LTBP1 or TGF- $\beta$ 1 expression proved a beneficial role in patients treated with anti-PD1  
57 immunotherapy, making unlikely a possible immunosuppressive role of LTBP1/TGF- $\beta$ 1 upon ERK5  
58 inhibition. This study, therefore, identifies additional desirable effects of ERK5 targeting, providing  
59 evidence of an ERK5-dependent tumor suppressive role of TGF- $\beta$  in melanoma.

## 60 **Introduction**

61 Malignant melanoma is one of the most aggressive skin cancers, and its incidence is increasing  
62 worldwide. Early-stage disease can be cured in the majority of cases by surgical excision, while late-  
63 stage melanoma is still a highly lethal disease [1]. Common genetic alterations associated with  
64 melanoma include mutations in BRAF (50-60%), NRAS (20-25%) and NF1 (14%) that hyperactivate  
65 the mitogen-activated protein kinase (MAPK) ERK1/2, thus supporting sustained cell proliferation  
66 [2]. Development of BRAF- and MEK1/2-targeting drugs and immunotherapy have greatly increased  
67 the survival of melanoma patients [3]. However, intrinsic or acquired resistance to the former as well  
68 as the lack of responsiveness to the latter limit the benefits of available therapies [4,5].

69 ERK5 (also referred to as big mitogen-activated protein kinase 1, BMK1), the last discovered  
70 member of conventional MAPKs, is involved in cell survival, proliferation and differentiation of  
71 several cell types [6], and plays a relevant role in the biology of cancer, including melanoma [7,8,9].  
72 ERK5 activation is achieved through MEK5-dependent or -independent phosphorylation that  
73 stimulates ERK5 nuclear translocation, a key event for cell proliferation [10,11]. On the other hand,  
74 a recent report showed that, upon ERK5 inhibition, melanoma cells undergo cellular senescence, and  
75 produce a number of soluble mediators (namely CXCL1, CXCL8 and CCL20) typically involved in  
76 the senescence-associated secretory phenotype (SASP) that slow down the proliferation of melanoma  
77 cells [9,12].

78 Accumulating evidence points to the involvement of transforming growth factor  $\beta$  (TGF- $\beta$ )  
79 in cellular senescence [13]. TGF- $\beta$  secretion and activation is regulated by its association to latent-  
80 transforming growth factor  $\beta$ -binding protein 1 (LTBP1) [14,15]. The roles normally played by  
81 TGF- $\beta$  signaling are to control proliferation, differentiation and other functions in most cells. These  
82 roles are highly context-dependent, and TGF- $\beta$  appears to induce even opposite effects in different  
83 contexts [16]. Regarding melanoma in particular, the role of tumor suppression versus tumor  
84 promotion of TGF- $\beta$  has been scarcely addressed [17]. This paper identified a tumor suppressive

85 role for LTBP1/TGF- $\beta$  among the antitumoral outcomes of ERK5 inhibition, that could be  
86 exploited for future therapeutic strategies in melanoma.

87

## 88 **Material & Methods**

### 89 *Cells and cell culture*

90 A375<sup>BRAFV600E</sup> (RRID:CVCL\_0132) [18] and SK-Mel-2<sup>NRASQ61R</sup> (RRID:  
91 CVCL\_0069CVCL\_0069) [19] melanoma cells were obtained from ATCC; SK-Mel-5<sup>BRAFV600E</sup>  
92 (RRID:CVCL\_0527) melanoma cells [19] were kindly provided by Dr. Laura Polisenò (CRL-ISPRO,  
93 Pisa, Italy); SSM2c melanoma cells have been described elsewhere [20]. Cells were maintained in  
94 DMEM with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L glutamine, 50 U/mL  
95 penicillin and 50 mg/mL streptomycin (Euroclone, Milano, Italy). Cell lines are authenticated yearly  
96 (BMR Genomics, Padua, Italy) by STR profiling using Promega PowerPlex Fusion System Kit  
97 (Promega Corporation, Madison, WI, USA). Presence of Mycoplasma was periodically tested by  
98 PCR.

99

### 100 *Drugs*

101 ERK5 inhibitors XMD8-92 [21] and JWG-071 [22] were from MedChemExpress (Monmouth  
102 Junction, NJ, USA). Cell cycle inhibitor L-mimosine was from Sigma-Aldrich (St Louis, MO, USA).

103

### 104 *Cell lysis and Western Blot*

105 Total cell lysates were obtained using Laemmli Buffer or RIPA buffer as reported previously  
106 [23]. Immunoprecipitation (IP) was performed by incubating 2 mg of CM proteins with the anti-TGF-  
107  $\beta$  antibody and 20  $\mu$ L of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, USA) for 24 hours  
108 at 4 °C. Immunocomplexes were then washed for three times and proteins eluted using Laemmli  
109 Buffer. Proteins were separated by SDS-PAGE and transferred onto Amersham Protran nitrocellulose

110 membranes (GE Healthcare, Chicago, IL, USA) by electroblotting. Infrared imaging (Odyssey, Li-  
111 Cor Bioscience, Lincoln, NE, USA) was performed. Images were quantified with ImageJ 1.53k  
112 software (<https://imagej.net/ij/>; Last access 18/01/2024). The list of the antibodies is in Table 1.

113

#### 114 *RNA interference*

115 A375 and SK-Mel-5 cells were transduced with control non-targeting shRNA (shNT) or  
116 ERK5-specific shRNAs (shERK5-1 and shERK5-2) (Table 2) as previously reported [23].  
117 Transduced cells were selected with 2 µg/mL puromycin for at least 72 hours. Fourteen days after  
118 lentiviral transduction, medium was replaced with DMEM/10% FBS, and CM were harvested after  
119 72 hours. For siRNA inhibition studies, the cells were transfected with human LTBP1 siRNAs  
120 (SASI\_Hs01\_00187276 and SASI\_Hs01\_00168991) or negative control siNT (SIC001) from Sigma-  
121 Aldrich at a final concentration of 100 nM using Lipofectamine 2000 reagent (Thermo Fisher  
122 Scientific, Waltham, MA, USA), following the manufacturer's instructions. 72 hours after  
123 transfection, cells were harvested for protein extraction and additional analysis.

124

#### 125 *Measurement of cell viability, cell cycle phase distribution and cell death*

126 The number of viable cells in culture was evaluated by counting trypan blue-positive and  
127 negative cells with a hemocytometer. Cell cycle phase distribution (propidium iodide staining) was  
128 estimated by flow cytometry using a FACSCanto (Beckton & Dickinson, San José, CA, USA) as  
129 previously reported [24]. Dead cells were evaluated by flow cytometry using a FACSCanto (Beckton  
130 & Dickinson). AnnexinV-positive and Annexin-V-negative/PI-positive cells were measured using  
131 Annexin-V-FLUOS Staining Kit (Sigma-Aldrich), as previously reported [24].

132

#### 133 *Transcriptomic analysis*

134 Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany), and mRNA  
135 expression was evaluated with Affymetrix Clariom-S Human Genechip following the manufacturer's

136 instructions. Transcriptome analysis console (TAC) software was used (fold change>1.5/<1.5 and  
137  $p \leq 0.05$ ) to identify differentially expressed genes (DEG).

138

### 139 *Patients*

140 Analysis of the relationship between LTBP1 and TGFB1 mRNA expression and overall  
141 survival (OS) of melanoma patients was carried out using the publicly available SKCM data set from  
142 The Cancer Genome Atlas (TCGA PanCancer Atlas) on cBioPortal for Cancer Genomics  
143 (<https://www.cbioportal.org>; last access: October 10th, 2023 [25,26]). The same database was used  
144 to verify the correlation between LTBP1 and TGFB1 mRNA. Analysis of the relationship between  
145 LTBP1 and TGFB1 mRNA expression and outcome OS and DFS of melanoma patients treated with  
146 anti-PD1 therapy was carried out using the open access database Kaplan-Meier plotter  
147 (<http://www.kmplot.com>; last access: October 10th, 2023 [27]). Expression of LTBP1 in normal,  
148 primary and metastatic tumors was obtained from TCGA dataset on TNMplot database  
149 (<http://www.tnmplot.com>; last access: October 10th, 2023 [28]).

150

### 151 *Quantitative real-time PCR (qPCR)*

152 Total RNA was isolated using TriFast II (Euroclone). cDNA synthesis was carried out using  
153 ImProm-II Reverse Transcription System, while qPCR was performed using GoTaq qPCR Master  
154 Mix (Promega Corporation). QPCR was performed using CFX96 Touch Real-Time PCR Detection  
155 System (Bio-Rad, Hercules, CA, USA). Expression levels were determined by qPCR with the  
156 primers: Forward: 5'-TGAATGCCAGCACCGTCATCTC-3' and reverse: 5'-  
157 CTGGCAAACACTCTTGTCTCC-3' for LTBP1. mRNA expression was normalized to: Forward:  
158 5'-GTCTCCTCTGACTTCAACAGCG-3' and reverse: 5'-ACCACCCTGTTGCTGTAGCCAA-3'  
159 GAPDH mRNA and: Forward: 5'-ACCCGTTGAACCCCATTCGTGA-3' and reverse: 5'-  
160 GCCTCACTAAACCATCCAATCGG-3' for 18S mRNA.

161

162 *Cell viability assay*

163 Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide  
164 (MTT) assay. Cells were seeded in 96-well plate in DMEM/10% FBS. After 24 hours, medium was  
165 replaced with CM and cells were further incubated for 72 hours. MTT (0.5 mg/mL) was added during  
166 the last 4 hours. Plates were read at 595 nm using a Microplate reader-550 (Bio-Rad). For  
167 neutralization experiments, control isotype IgG or neutralizing antibodies (Table 1) were added to  
168 CM prior to administration to cells.

169

170 *Immunohistochemistry*

171 Formalin-fixed paraffin-embedded sections from archival xenografts established with A375  
172 cells from XMD8-92 (25 mg/kg)- or vehicle (2-hydroxypropyl- $\beta$ -cyclodextrin 30%)-treated mice  
173 were used [8]. Experiments had been approved by the Italian Ministry of Health (authorization no.  
174 213/2015-PR) and were in accordance with the Italian ethic guidelines and regulations. Sections (3  
175  $\mu$ m thick) were deparaffinized and were incubated overnight at 4°C with primary antibodies (Table  
176 2) and 3,3'-diaminobenzidine (DAB; Thermo Fisher Scientific) used as a chromogen. Sections were  
177 counterstained with hematoxylin and the percentage of stained area was evaluated with ImageJ 1.53k  
178 software. Representative photographs are shown (original magnification, 40X). Scale bar, 100  $\mu$ m.

179

180 *Invasion assay*

181 A375 or SK-Mel-5 melanoma cells ( $1 \times 10^4$  cells/well) were seeded in DMEM supplemented  
182 with 10% Bovine serum albumin (BSA), in the presence or absence of neutralizing antibodies onto  
183 the top chamber of 48-well transwell plates equipped with 8  $\mu$ m polycarbonate nucleopore filters  
184 (Neuro Probe, Gaithersburg, MD, USA) pre-coated with Matrigel (Sigma-Aldrich). The bottom  
185 chamber was supplemented with CM obtained as described above. After 24 hour-incubation cells that  
186 had not migrated were removed with a cotton swab from the upper surface of filters and cells that had  
187 migrated to the lower surface of the membrane were subjected to Diff-Quick staining (Medion

188 Diagnostics AG, Duding, Switzerland) and observed with a light microscope. The number of cells  
189 per well was evaluated by counting cells in 5 randomly chosen microscope fields (20X  
190 magnification).

191

#### 192 *Statistical analysis*

193 Data represent mean or  $\pm$  SD values calculated on at least three independent experiments. P  
194 values were calculated using Student t-test or one-way ANOVA (multiple comparison).  $P < 0.05$  was  
195 considered statistically significant.

196

## 197 **Results**

### 198 *ERK5 inhibition determines an increase of LTBP1*

199 CXCL1, CXCL8 and CCL20 have been recently identified among the SASP-related soluble  
200 mediators that are responsible for the reduced proliferation in BRAFV600E melanoma cells  
201 undergoing cellular senescence following ERK5 KD [9]. In view of the exploitation of ERK5  
202 targeting for the treatment of melanoma, further characterization of the secretome of BRAFV600E  
203 melanoma cells upon ERK5 inhibition was performed, taking advantage of a previously performed  
204 transcriptomic analysis in A375 and SK-Mel-5 ERK5-KD cells [9]. Using this approach, it emerged  
205 that ERK5 KD upregulated LTBP1 mRNA levels, when compared to control non-targeting shRNA-  
206 transduced cells (Supplementary Figure S1). QPCR confirmed the increased expression of LTBP1  
207 mRNA upon ERK5 KD in both A375 and SK-Mel-5 BRAFV600E cells (Figure 1A). Interestingly,  
208 publicly available Skin Cutaneous Melanoma (SKCM) data set from TCGA on TNMplot [28]  
209 provided evidence that LTBP1 mRNA levels are lower in primary and metastatic melanomas than in  
210 normal tissues from non-cancer patients (Figure 1B). However, the same data set did not provide  
211 evidence of changes in ERK5 mRNA along melanoma progression (not shown), in keeping with a  
212 previous report showing consistent activation of the MEK5/ERK5 pathway without appreciable  
213 ERK5 overexpression in melanoma patients [8]. More importantly, using the same dataset on



214 cBioPortal for Cancer Genomics it emerged that higher expression levels of LTBP1 mRNA are  
215 associated with a better overall survival (OS) (Figure 1C) of melanoma patients, pointing to a possible  
216 tumor suppressive role of LTBP1 in melanoma. ERK5 KD resulted in increased levels of LTBP1  
217 protein (Figure 1D), and the same effects were recapitulated by pharmacological inhibition of ERK5  
218 using XMD8-92 [21] and the more specific JWG-071 [22] small molecule inhibitors (Figure 1E).  
219 Effectiveness of the ERK5 inhibitors was confirmed by the reduced protein level of the downstream  
220 target KLF2 [29]. Of note, AX15836 that inhibits the catalytic function of ERK5 but paradoxically  
221 stimulates its transactivation function [30,31] did not elicit the same effects (not shown). Taken  
222 together, the above data indicate that ERK5 negatively regulates LTBP1, whose expression correlates  
223 with a better outcome in melanoma patients.

224

225 *ERK5 inhibition promotes an LTBP1-dependent increase of TGF- $\beta$ 1, whose expression is associated*  
226 *with a better prognosis in melanoma*

227 Because LTBP1 is involved in the stabilization and activation of TGF- $\beta$ , which plays a  
228 relevant role in cancer onset and progression [15], the impact of ERK5 KD on TGF- $\beta$  protein levels  
229 was analyzed. Increased protein levels of both mature (Figure 2A) and latent forms (Supplementary  
230 Figure S2A) of TGF- $\beta$ 1 were found, in conditioned media (CM) and whole cell lysates, respectively,  
231 of ERK5-KD A375 and SK-Mel-5 cells. Of note, mRNA levels of TGFB1 were not consistently  
232 affected (i.e. were not increased in both cell lines upon ERK5 KD), pointing to post-transcriptional  
233 effects of LTBP-1 on TGF- $\beta$  upon ERK5 KD (Supplementary Figure S2B), at least in our  
234 experimental models. Interestingly, in line with the fact that LTBP-1-dependent regulation of TGF- $\beta$   
235 could impact the activity of transcription factors (e.g. SMAD proteins, AP-1, NF- $\kappa$ B, and SP1  
236 [32,33,34]) known to be regulated by TGF- $\beta$  itself that are, in turn, able to regulate TGF- $\beta$  expression,  
237 the SKCM data set from TCGA on cBioPortal provided evidence of a positive correlation (Spearman:  
238 0.32,  $p = 2.82e-12$ ) between LTBP1 mRNA and that of TGFB1 (Figure 2B). More importantly, the  
239 same dataset provided a positive association between higher levels of TGFB1 expression and a better

240 prognosis in melanoma patients (Figure 2C). To prove that LTBP1 participates in the regulation of  
241 TGF- $\beta$ 1 protein level in melanoma cells, LTBP1 was KD using two different siRNAs (Figure 2D).  
242 LTBP1 KD determined a marked decrease of TGF- $\beta$ 1 protein in both A375 and SK-Mel-5 cells  
243 (Figure 2E), and prevented the increase of TGF- $\beta$ 1 upon pharmacological inhibition of ERK5  
244 (Supplementary Figure S2C). Importantly, both LTBP1 and TGF- $\beta$ 1 protein levels were increased  
245 upon ERK5 inhibition *in vivo*. Indeed, administration of the ERK5 inhibitor XMD8-92, which had  
246 been previously shown to reduce melanoma tumor growth similarly to ERK5 KD [8], induced a  
247 robust increase of both LTBP1 and TGF- $\beta$ 1 in A375 xenografts, with respect to vehicle-treated mice  
248 (Figure 2F).

249

250 *TGF- $\beta$ 1 exerts an antiproliferative effect in melanoma cells upon ERK5 KD*

251 It has been shown that ERK5 KD results in the increased production of CXCL1, CXCL8 and  
252 CCL20 in melanoma cells, and that these chemokines are responsible for a reduced viability of  
253 melanoma cells [9]. TGF- $\beta$  is involved in cellular senescence and is able to exert potent growth  
254 inhibitory activities in various cell types and in different context, including cancer cells [13]. Along  
255 this line, here TGF- $\beta$ 1 emerges to be among the soluble factors responsible for a reduced viability of  
256 melanoma cells upon ERK5 KD. Indeed, TGF- $\beta$ 1-neutralizing antibodies prevented in a dose-  
257 dependent manner the anti-proliferative effect induced by CM harvested from ERK5-KD A375  
258 (Figure 3A) or SK-Mel-5 (Figure 3B) cells. The above effects were not restricted to BRAFV600E-  
259 mutated melanoma cells. Indeed, in both N-RAS-mutated SK-Mel-2 and triple wild type SSM2c  
260 melanoma cells, TGF- $\beta$ 1-neutralizing antibodies reverted the reduction of cell proliferation elicited  
261 by the ERK5-KD-derived CM (Supplementary Figure S3A). Moreover, in keeping with the biological  
262 evidence, both pharmacological and genetic inhibition of ERK5 determined an increase of TGF- $\beta$ 1  
263 in these cell lines (Supplementary Figure S3B). To confirm that TGF- $\beta$ 1 exerts an antiproliferative  
264 effect in melanoma cells, A375 and SK-Mel-5 cells were treated with human recombinant TGF- $\beta$ 1.

265 This cytokine reduced the number of viable cells in culture in a dose-dependent manner in both cell  
266 lines (Figure 3C). This effect was maximal with 100 ng/ml, a concentration in line with previous  
267 reports [35,36]. In order to deepen how TGF- $\beta$ 1 affects cell growth, cell-cycle analysis was  
268 performed, and showed that treatment with TGF- $\beta$ 1 significantly increased the fraction of cells in  
269 G0/G1 phase (Figure 3D). In the same experimental settings, TGF- $\beta$ 1 determined the increase of the  
270 cyclin dependent kinase inhibitor p21 (Supplementary Figure S3C). In further support of a possible  
271 involvement of p21 in the antiproliferative effects of TGF- $\beta$ 1 upon ERK5 inhibition, the treatment  
272 with TGF- $\beta$ 1-neutralizing antibodies was able to reduce the increase of p21 elicited by CM harvested  
273 from ERK5-KD (A375) cells in both A375 and SK-Mel-5 cell lines (Supplementary Figure S3D).  
274 The reduction of cell number observed in melanoma cells treated with TGF- $\beta$ 1 was partially due to  
275 increased cell death (Figure 3E). On the whole, the above data provides evidence that TGF- $\beta$ 1 is  
276 among the soluble mediators that increase upon ERK5 inhibition, and is then responsible for the  
277 reduced proliferation.

278

279 *TGF- $\beta$ 1 produced upon ERK5 inhibition reduces the invasive ability of melanoma cells*

280 The possible impact of the secretome of ERK5-KD cells on melanoma cell invasiveness was  
281 then tested. CM from ERK5-KD cells markedly reduced the invasive ability of A375 and SK-Mel-5  
282 cells (Supplementary Figure S4A), in the presence of mimosine, a DNA replication inhibitor used at  
283 concentration able to completely prevent changes in the number of cells along the duration (i.e. 24  
284 hours) of the invasion assays (Supplementary Figure S4B and C). To shed light on the possible role  
285 of TGF- $\beta$ 1 in the regulation of this biological process upon ERK5 KD, the effect of TGF- $\beta$ 1  
286 neutralizing antibodies on cell invasion ability was evaluated. TGF- $\beta$ 1 neutralizing antibodies were  
287 able to restore A375 (Figure 4A) and SK-Mel-5 (Figure 4B) invasion ability reduced by CM harvested  
288 from ERK5-KD A375 or SK-Mel-5 cells, while control IgG did not. To confirm that TGF- $\beta$ 1 is able  
289 to reduce melanoma cell invasiveness, A375 and SK-Mel-5 cells were treated with increasing doses

290 of this cytokine. TGF- $\beta$ 1 dose-dependently decreased the invasive ability of both A375 and SK-Mel-  
291 5 cells (Figure 4C and D). Altogether, the above data indicate that TGF- $\beta$ 1 reduces the invasive  
292 propensity of melanoma cells, at least *in vitro*.

293

294 *Increased TGF- $\beta$ 1 and LTBP1 expression positively affects the impact of immunotherapy in*  
295 *melanoma patients*

296 As reported above, higher levels of both TGF- $\beta$ 1 and LTBP1 correlate with a better OS  
297 (Figure 1C and 2C). Moreover, OS and disease-free survival (DFS) of patients treated with anti-PD1  
298 therapy (i.e. Nivolumab or Pembrolizumab) are significantly higher in patients with high TGF- $\beta$ 1  
299 expression than in those with lower expression (Figure 5A). This positive association was also  
300 detected regarding high levels of LTBP1 expression and better OS and DFS in melanoma patients  
301 treated with anti-PD1 therapy (Figure 5B), pointing to additional desirable effects of ERK5 inhibition  
302 in melanoma.

303

## 304 **Discussion**

305 TGF- $\beta$  controls a wide spectrum of cellular functions, and deregulated TGF- $\beta$  signaling has  
306 been linked to several human diseases, including cancer [37]. In particular, TGF- $\beta$  may play a double-  
307 edged sword role in tumor progression [38,39], acting as a tumor suppressor during the early stage of  
308 the tumor, since inhibition of TGF- $\beta$  signaling results in the disruption of normal homeostatic process  
309 and subsequent carcinogenesis, while behaving as a tumor promoter at later stages [40].  
310 Understanding how TGF- $\beta$ 1 can coordinate its effects in melanoma is a key issue in the biology of  
311 this cancer.

312 ERK5 has been recently reported to be involved in melanoma growth [8], and ERK5 inhibition  
313 induces marked cellular senescence and production of several soluble mediators involved in the SASP  
314 in both BRAF-mutated and -wild-type melanoma cells and xenografts [9]. In this study, ERK5  
315 inhibition evokes an increased expression of LTBP1, which is known to modulate the availability of

316 TGF- $\beta$ 1 [15]. Along this line, besides increased LTBP1 expression, increased TGF- $\beta$ 1 protein levels  
317 were found in ERK5-KD melanoma cells, and in A375 xenografts from XMD8-92-treated mice.  
318 LTBP1 resulted to be responsible for the regulation of TGF- $\beta$ 1 protein levels, likely through a post  
319 transcriptional regulation, and also to prevent the increase on the latter upon ERK5 inhibition. This  
320 work also identifies an anti-proliferative and anti-invasiveness ability of TGF- $\beta$ 1 in melanoma cells,  
321 providing evidence that the increase of LTBP1/TGF- $\beta$ 1 complex could be an additional desirable  
322 effect obtained by ERK5 inhibition.

323 TGF- $\beta$  is a potent inhibitor of cell proliferation, which is thought to result from its ability to  
324 induce G1 cell cycle arrest [41]. In line with this fact, the data provided in this work indicate that  
325 TGF- $\beta$ 1 is among the soluble factors responsible for the reduction of melanoma cell proliferation  
326 induced by the secretome of ERK5-KD melanoma cells. In fact, this event is partially restored by  
327 TGF- $\beta$ 1-neutralizing antibodies. Moreover, in BRAFV600E-expressing cells, TGF- $\beta$ 1 slows down  
328 cell cycle progression with the accumulation of cells in G0/G1 phase, and is able to increase cell  
329 death. Despite these effects are elicited at relatively high TGF- $\beta$ 1 concentrations, the latter are in line  
330 with previous reports [35,36], and are consistent with the amount contained in the CM of ERK5-KD  
331 melanoma cells. The observed antiproliferative effects are consistent with the results obtained in other  
332 studies, which demonstrated that cell cycle arrest was induced upon treatment with TGF- $\beta$ 1 via  
333 SMAD2/3 in proliferating melanoma cells *in vitro* and *in vivo* [42,43,44]. Moreover, in another paper,  
334 the activation of TGF- $\beta$ 1 led to the upregulation of PAI-1 expression that resulted in tumor growth  
335 inhibition in murine melanoma [45]. The above results, including those described in this manuscript,  
336 are at variance with a previous report showing that inhibition of canonical TGF- $\beta$  signaling inhibited  
337 tumor growth in melanoma [46]. Despite here it clearly emerges an oncopressive role for TGF- $\beta$ 1  
338 in melanoma cells upon ERK5 inhibition, the molecular mechanism underlying this connection  
339 remains to be established. However, TGF- $\beta$ 1 was found to increase the expression of the cyclin

340 dependent kinase inhibitor p21, a previously established ERK5-regulated protein [8,9], that is a  
341 downstream mediator of the antiproliferative effects of TGF- $\beta$ , including in melanoma cells [38,47].

342 Another interesting finding of this study is the demonstration that ERK5-KD melanoma  
343 cells produce TGF- $\beta$ 1, which exerts an anti-invasive capacity. These results, together with the  
344 identified antiproliferative effect, are in keeping with the evidence reported here that melanoma  
345 patients with higher expression of TGF- $\beta$ 1 have a better prognosis. On the other hand, they are at  
346 variance with the established notion that, at least in the advanced stages, TGF- $\beta$  acts as a tumor  
347 promoter by stimulating invasiveness along the epithelial to mesenchymal transition [48]. Of note,  
348 A375 and SK-Mel-5 cell lines used as models for this study were derived from metastatic  
349 melanoma [18,19]. Moreover, elevated expression levels of TGF- $\beta$ 1 have been associated with  
350 melanoma progression *in vivo*, and TGF- $\beta$ 1-elicited signals have been reported to stimulate  
351 melanoma cell dissemination from primary tumors [49,50].

352 From the clinical point of view, the possibility to elicit an increase in LTBP1 and TGF- $\beta$ 1  
353 expression following ERK5 inhibition seems to have positive therapeutic implications in melanoma  
354 patients. Indeed, LTBP1 expression is lower in primary and metastatic melanoma compared to  
355 healthy tissues, and melanoma patients with higher expression of LTBP1 or TGF- $\beta$ 1 have a better  
356 prognosis (OS) with respect to those with lower ones. On the other hand, *in silico* data analysis  
357 revealed that among melanoma patients that have received anti-PD1 antibodies, those with higher  
358 expression of LTBP1 or TGF- $\beta$ 1 showed improved OS or DFS compared to those with low  
359 expression. This fact is of relevance, taking into consideration that TGF- $\beta$  affects multiple  
360 components of the immune system, exerting most of times systemic immune suppression [51].  
361 Furthermore, the first-line therapeutic approach for advanced melanoma consists in immunotherapy  
362 with anti-PD1 antibodies (Nivolumab/Pembrolizumab) or targeted therapy with BRAF and MEK  
363 inhibitors, and their combination is under study [52]. Based on all above, targeting ERK5 is also  
364 expected to boost the efficacy of immunotherapy in melanoma patients, adding value to the possible

365 targeting of ERK5 in this cancer, taking into consideration that ERK5 inhibition has been reported  
366 to reduce melanoma growth and to improve BRAF targeting *in vivo* [8], and that ERK5 activation is  
367 among the resistance mechanism to RAF-MEK1/2-ERK1/2 directed therapies [9].

368

#### 369 **Authors' Contributions**

370 AT: Data curation, formal analysis, investigation, writing–original draft, writing–review and editing.

371 AM, ZL: Data curation, formal analysis, methodology, writing–review. IT: Data curation, formal

372 analysis and editing. TG: formal analysis, methodology. AE-O, AP, BS Resources and editing. ER:

373 Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition,

374 investigation, writing–original draft, project administration, writing–review and editing.

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518 **Figure Legends**519 **Figure 1. Effects of ERK5 inhibition on the expression of LTBP1 in melanoma cells. A)**

520 A375 and SK-Mel-5 cells transduced with lentiviral vectors harboring control non-targeting shRNA

521 (shNT) or ERK5-specific shRNAs (shERK5-1 and shERK5-2) were lysed after 72 hours, and LTBP1

522 mRNA levels determined by qPCR. Data shown are means ( $\pm$  SD) from three independent523 experiments. \*\*,  $p < 0.01$  vs shNT. **B)** Violin plots show LTBP1 gene expression profile in normal

524 skin (Normal), primary (Tumor) and metastatic (Metastatic) melanoma obtained by SKCM data set

525 (TCGA) on TNMplot. \*\*\*\*,  $p < 0.0001$ . **C)** Kaplan-Meier analysis of the relationship between LTBP1

526 expression and overall survival (OS) in melanoma patients using the SKCM data set (TCGA) on

527 cBioPortal. Patients were stratified according to low or high LTBP1 expression. Median LTBP1

528 expression value was used as cut-off. In order to reduce noise, 5% of samples above and below the

529 cut-off value were excluded from the analysis ( $n=423$ , with  $n=223$  and  $n=200$  in the low/high group,530 respectively). HR = hazard ratio;  $HR < 1$  indicates reduced hazard of death. **D)** A375 and SK-Mel-5

531 cells transduced with control shNT or shERK5 (shERK5-1 and shERK5-2) were lysed after 72 hours.

532 Western Blot was performed with the indicated antibodies. Images are representative of three

533 independent experiments showing similar results. Migration of molecular weight markers is indicated

534 on the left (kDa). The graphs show average relative integrated density (RID)  $\pm$  SD of ERK5 protein535 levels normalized for tubulin content from three independent experiments. \*,  $p < 0.05$ , \*\*,  $p < 0.01$  vs536 shNT. **E)** A375 and SK-Mel-5 cells treated with XMD8-92 (5  $\mu$ M) or JWG-071 (5  $\mu$ M) for 72 hours

537 were lysed. Western Blot was performed with the indicated antibodies. Images are representative of

538 three independent experiments showing similar results. Migration of molecular weight markers is

539 indicated on the left (kDa). The graphs show average relative integrated density (RID)  $\pm$  SD of LTBP1540 protein levels normalized for tubulin content from three independent experiments. \*,  $p < 0.05$ , \*\*,  $p$ 541  $< 0.01$  vs Vehicle.

542

543 **Figure 2. Effects of ERK5 inhibition on TGF- $\beta$ 1 expression in melanoma cells and in**  
544 **xenografts. A)** TGF- $\beta$ 1 immunoprecipitation was performed in 72-hour-conditioned media (CM)  
545 from A375 or SK-Mel-5 cells transduced with shNT or shERK5 (shERK5-1 or shERK5-2) lentiviral  
546 vectors. Human recombinant TGF- $\beta$ 1 (100 ng/ml) was used as positive control. Western Blot was  
547 performed with the indicated antibodies. Images are representative of three independent experiments  
548 showing similar results. Migration of molecular weight markers is indicated on the left (kDa). The  
549 graphs show average relative integrated density (RID)  $\pm$  SD of TGF- $\beta$ 1 protein levels normalized for  
550 IgG content from three independent experiments. \*,  $p < 0.05$ , \*\*,  $p < 0.01$  vs shNT. **B)** Expression  
551 levels of TGF $\beta$ 1 and LTBP1 mRNA from SKCM dataset (TCGA) on cBioPortal. **C)** Kaplan-Meier  
552 analysis of the relationship between TGF $\beta$ 1 expression and overall survival (OS) in melanoma  
553 patients using SKCM dataset (TCGA) on cBioPortal. Patients were stratified according to low or high  
554 *TGF $\beta$ 1* expression, using median TGF $\beta$ 1 expression value as cut-off. In order to reduce noise, 10%  
555 of samples above and below the cut-off value were excluded from the analysis (n=376, with n=195  
556 and n=181 in the low/high group, respectively). HR= hazard ratio; HR < 1 indicates reduced hazard  
557 of death. **D)** QPCR of LTBP1 mRNA from LTBP1-KD A375 and SK-Mel-5 cells following treatment  
558 with LTBP1-targeting (siLTBP1-1 or siLTBP1-2) siRNAs or control non-targeting siRNAs (siNT)  
559 for 72 hours. Data shown are mean ( $\pm$ SD) of three independent experiments. \*,  $p < 0.05$ , \*\*,  $p < 0.01$   
560 vs siNT. **E)** Western Blot showing TGF- $\beta$ 1 protein levels in LTBP1 KD-cells 72 hours after  
561 transfection with LTBP1-targeting siRNA (siLTBP1-1 or siLTBP1-2) or control non-targeting  
562 siRNA (siNT). Migration of molecular weight markers is indicated on the left (kDa). The graphs  
563 show average relative integrated density (RID)  $\pm$  SD of TGF- $\beta$ 1 protein levels normalized for tubulin  
564 content from three independent experiments. \*,  $p < 0.05$  vs siNT. **F)** IHC detection of LTBP1 (left) or  
565 TGF- $\beta$ 1 (right) in XMD8-92 (25 mg/kg)- or vehicle (2-hydroxypropyl- $\beta$ -cyclodextrin 30%)-treated  
566 mice [8]. Hematoxylin counterstaining was performed. Bar plots of percentage (%) of LTBP1 or  
567 TGF- $\beta$ 1-positive cells are shown. The percentage of positive cells was calculated from six different  
568  $\times 40$  magnified fields from three randomly chosen vehicle and XMD8-92-treated tumors.

569 Representative photographs are shown (original magnification,  $\times 40$ ). Scale bar, 100  $\mu\text{m}$ . \*,  $p < 0.05$   
570 vs Vehicle.

571

572 **Figure 3. Involvement of TGF- $\beta 1$  in the anti-proliferative outcome of ERK5 inhibition**  
573 **in melanoma cells. A-B)** MTT performed in A375 and SK-Mel-5 cells treated for 72 hours with 72-  
574 hour conditioned media (CM), obtained from A375 or SK-Mel-5 cells transduced with shNT or  
575 shERK5 (shERK5-1 or shERK5-2) lentiviral vectors, alone or in combination with the indicated  
576 concentrations of TGF- $\beta 1$  neutralizing antibodies (TGF- $\beta 1$  Neu-Ab). Data shown are means ( $\pm$  SD)  
577 from three independent experiments. \*,  $p < 0.05$ , \*\*,  $p < 0.01$  vs shNT CM; §,  $p < 0.05$ , §§,  $p < 0.01$  vs  
578 shERK5-1 CM; #,  $p < 0.05$  vs shERK5-2 CM. **C)** Cells were treated with the indicated concentrations  
579 of TGF- $\beta 1$  for 72 hours, and the number of viable cells was counted. Histograms represent means  
580 ( $\pm$ SD) from three independent experiments. \*\* $p < 0.01$  vs untreated. **D)** Cells were treated or not with  
581 100 ng/ml human recombinant TGF- $\beta 1$  for 72 hours, and cell-cycle phase distribution was then  
582 determined. Data shown are means  $\pm$  SD from three independent experiments. \* $p < 0.05$  vs untreated.  
583 **E)** Dead cells (Annexin-V-positive and Annexin-V-negative/PI-positive cells) were evaluated after  
584 treating A375 or SK-Mel-5 melanoma cells with or without 100 ng/ml of human recombinant TGF-  
585  $\beta 1$ . Histograms represent mean percentages  $\pm$  SD from three independent experiments. \* $p < 0.05$  vs  
586 untreated.

587

588 **Figure 4. Involvement of TGF- $\beta 1$  in the anti-invasive effect of ERK5 inhibition in**  
589 **melanoma cells. A-B)** Invasion assays were performed for 24 hours in A375 (**A**) and SK-Mel-5 (**B**)  
590 cells in the presence of 72-hour CM, obtained from A375 or SK-Mel-5 cells transduced with shNT  
591 or shERK5 (shERK5-1 or shERK5-2) lentiviral vectors, alone or with TGF- $\beta 1$  neutralizing antibodies  
592 (TGF- $\beta 1$  Neu-Ab, 10  $\mu\text{g}/\text{ml}$ ) or control IgG. Histograms represent means ( $\pm$  SD) from three  
593 independent experiments. \*\* $p < 0.01$  vs shNT CM/0, ### $p < 0.01$  vs shERK5-1 CM/0, §§ $p < 0.01$  vs  
594 shERK5-2 CM/0; ns: not significant. Representative pictures of wells treated as above are included.



595 Scale bar, 150  $\mu\text{m}$ . **C-D**) A375 and SK-Mel-5 cells were exposed for 24 hours at increasing  
596 concentrations of human recombinant TGF- $\beta$ 1. Histograms represent means ( $\pm$  SD) from three  
597 independent experiments. \*\* $p < 0.01$  vs NT.

598

599 **Figure 5. Impact of LTBP1 and TGF- $\beta$ 1 expression on the overall survival and disease-**  
600 **free survival in anti-PD1-treated melanoma patients. A)** 60 months follow-up Kaplan-Meier  
601 analysis of the relationship between TGF- $\beta$ 1 expression and overall survival (OS) in anti-PD1-treated  
602 melanoma patients (n=325) from Kaplan-Meier plotter database. Patients were stratified according to  
603 low or high TGF- $\beta$ 1 expression. The number of patients at risk in the low and high expression groups  
604 are indicated. **B)** 60 months follow up Kaplan-Meier analysis of the relationship between TGF- $\beta$ 1  
605 expression and DFS in melanoma anti-PD1 treated patients (n=234) calculated as in A. **C)** 60 months  
606 follow up Kaplan-Meier analysis of the relationship between LTBP1 expression and OS in melanoma  
607 anti-PD1 treated patients (n=325) calculated as in A. **D)** 60 months follow up Kaplan-Meier analysis  
608 of the relationship between LTBP1 expression and DFS in melanoma anti-PD1 treated patients  
609 (n=200) calculated as in A.

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621 **Table 1.** List of the antibodies used and their application.  
 622 WB: Western Blot; IHC: Immunohistochemistry; N: Neutralization.  
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ERK5	WB	Rabbit polyclonal	#3372	Cell Signaling Technology, USA
LTBP1	WB, IHC	Mouse monoclonal	sc-271140	Santa Cruz Biotechnology, USA
$\alpha$ -Tubulin	WB	Mouse monoclonal	sc-32293	Santa Cruz Biotechnology, USA
TGF- $\beta$	WB	Rabbit polyclonal	#3711	Cell Signaling Technology, USA
TGF- $\beta$ 1	N, IHC	Mouse monoclonal	69012-1-Ig	Proteintech Group, Inc, USA
KLF-2	WB	Rabbit monoclonal	#15306	Cell Signaling Technology, USA
IgG1	N	Mouse monoclonal	MAB002	R&D Systems, Inc. USA

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644 **Table 2.** List and sequences of the shRNA. \*Sequence reference from  
 645 <https://www.ncbi.nlm.nih.gov/gene/5598>

Gene	Sequence reference*	shRNA	Clone ID	Sequence
none	none	shNT	SHC202	5'- CCGGCAACAAGATGAAGAGCACCAACTCG AGTTGGTGCTCTTCATCTTGTTGTTTT-3'
<i>MAPK7</i>	NM_139032.X	shERK5-1	TRCN00 00010262	5'-CCGGGCTGCCCTGCTCAAGTCTTTG CTCGAGCAAAGACTTGAGCAGGGC AGCTTTTT-3'
<i>MAPK7</i>	NM_139032.X	shERK5-2	TRCN00 00010275	5'-CCGGGCCAAGTACCATGATCCTGA TCTCGAGATCAGGATCATGGTACTT GGCTTTTT-3'

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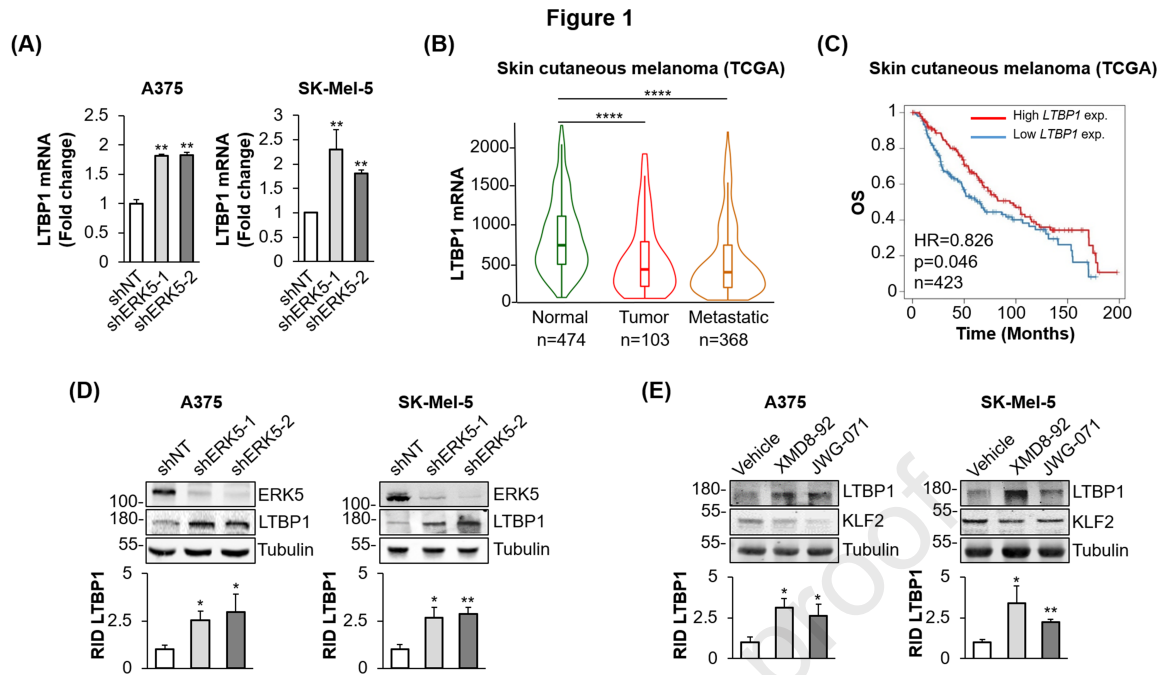


Figure 2

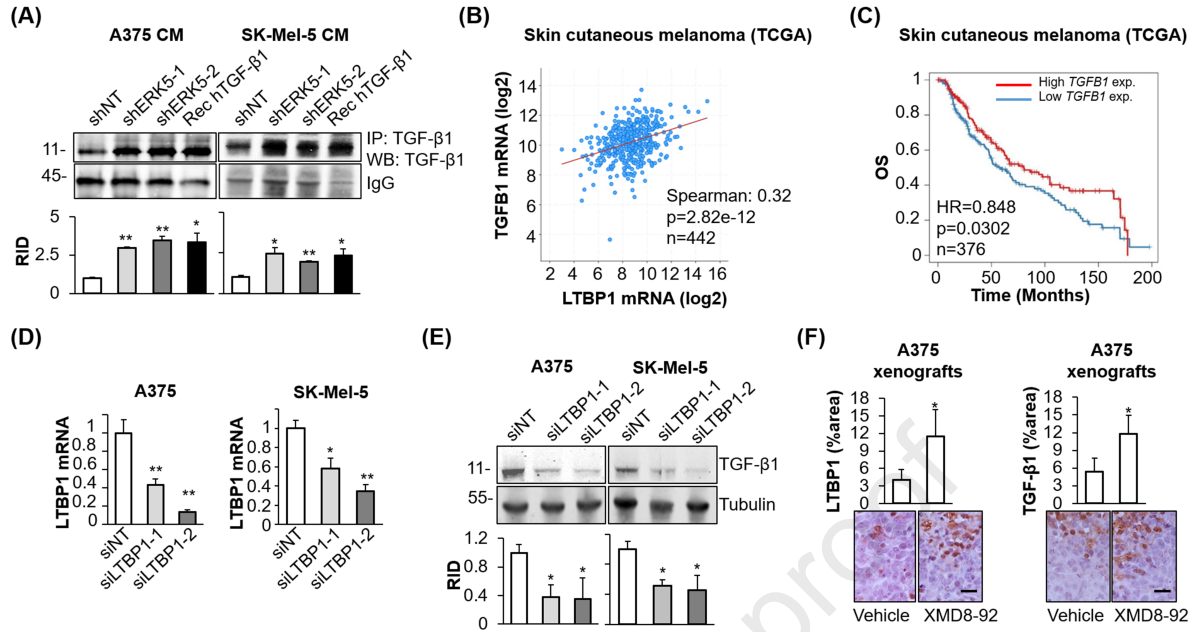


Figure 3

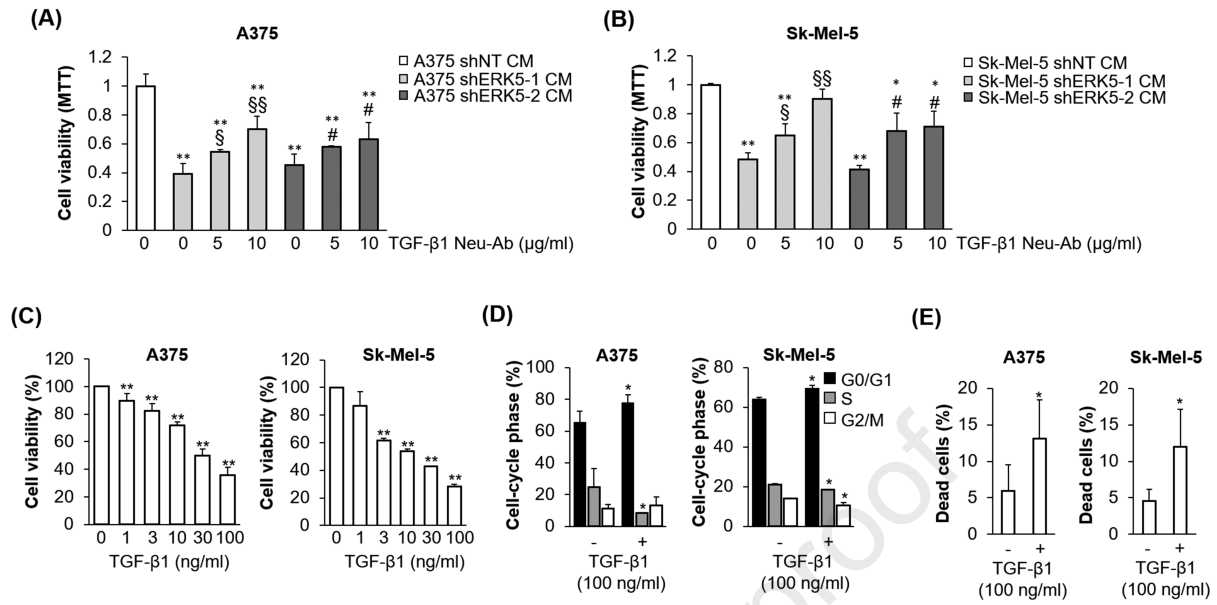


Figure 4

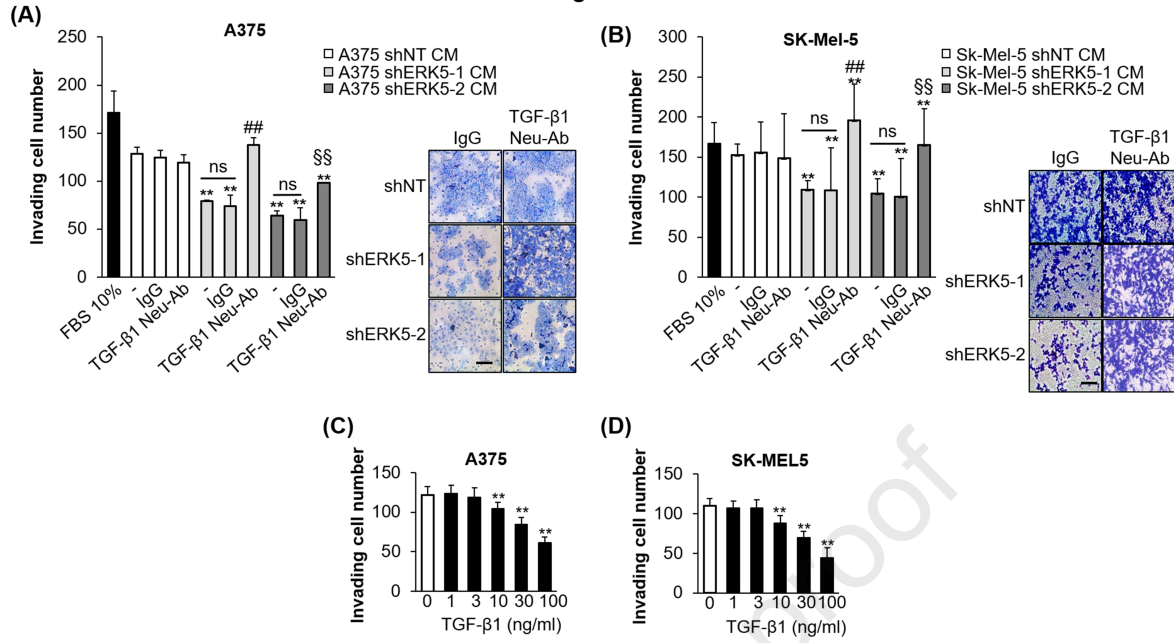
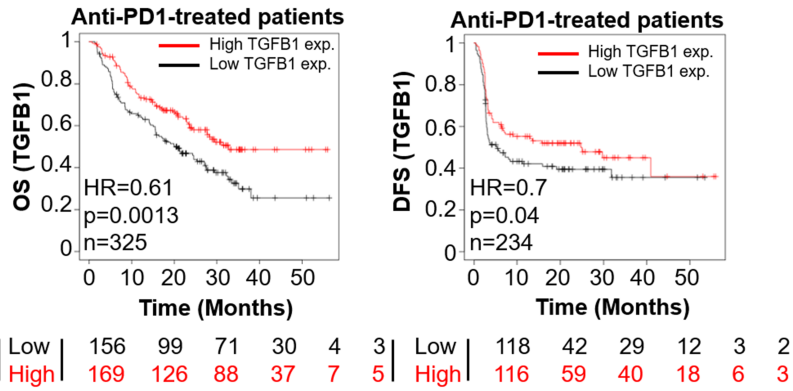


Figure 5

(A)



(B)

